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Expression of X-linked Genes in Deceased Neonates and Surviving Cloned Female Piglets

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Abstract

Animal cloning through somatic cell nuclear transfer is very inefficient, probably due to insufficient reprogramming of the donor nuclei, which in turn would cause the dysregulation of gene expression. X-Chromosome inactivation (XCI) is a multi-step epigenetic process utilized by mammals to achieve dosage compensation in females. Our aim was to determine if any dysregulation of X-linked genes, which would be indicative of unfaithful reprogramming of donor nuclei, was present in cloned pigs. Real time reverse transcription polymerase chain reaction (RT-PCR) was performed to quantify the transcript levels of five X-linked genes, *XIST*, *TSIX*, *HPRT1*, *G6PD*, *ARAF1* and one autosomal gene, *COL4A1* in major organs of neonatal deceased and surviving female cloned pigs and age-matched control pigs from conventional breeding. Aberrant expression level of these genes was prevalent in the neonatal deceased clones, while it was only moderate in cloned pigs that survived after birth. These results suggest a correlation between the viability of the clones and the normality of their gene expression and provide a possible explanation for the death of a large portion of cloned animals around birth.

Keywords

Gene expression; Porcine; X chromosome; nuclear transfer

Introduction

Since the creation of Dolly, the first mammal cloned from a differentiated adult somatic cell, production of live offspring through nuclear transfer (NT) of somatic cells has been successful in a variety of species, ranging from small laboratory mice to large domestic animals (Betthausen et al. 2000; Cibelli et al. 1998; Galli et al. 2003; Kato et al. 1998; Kubota et al. 2000; Onishi et al. 2000; Polejaeva et al. 2000; Senda et al. 2004; Shin et al. 2002; Wilmut et al. 1997). Somatic cell nuclear transfer revealed the extraordinary ability of the oocyte to reprogram the differentiated somatic genome to a totipotent state (Eggan et al. 2004;

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Humpherys et al. 2002). However, little is known about this reprogramming process, and the overall success rate of animal cloning using somatic cells remains very low across different animal species, likely due to insufficient epigenetic reprogramming. Faulty or incomplete epigenetic reprogramming has been observed in different stages of development (Cezar et al. 2003; Kang et al. 2001b; Mann et al. 2003). Even in cloned animals that survive to term, different aspects of epigenetic abnormalities are evident, although the majority of these animals appear to be healthy and normal (Archer et al. 2003; Li et al. 2004; Xue et al. 2002).

Pigs have been regarded as promising donors for xenotransplantation and attempts have been made to genetically modify the porcine genome through somatic cell NT in order to make the porcine organs immunologically more compatible to human (Dai et al. 2002; Kolber-Simonds et al. 2004; Lai et al. 2002; Ramsoondar et al. 2003). The production of pigs through NT has been particularly difficult, probably due to the inefficiencies of in vitro oocyte maturation and embryo culture, and the necessity for at least four good-quality embryos to establish a pregnancy. Consequently, few cloned offspring are available for comparison of gene expression to control pigs from conventional reproduction. To date, there has been only one report examining epigenetic reprogramming in full-term cloned pigs; Archer et al. found abnormal CpG methylation in a repetitive region in adult clones. Despite the availability of various studies on cloned mice and bovine calves, epigenetic reprogramming patterns found in one species may not be the same in others. For instance, the abnormal DNA demethylation patterns observed in cloned bovine and mouse embryos were absent in cloned porcine embryos (Kang et al. 2001b).

The mammalian X chromosome contains more than 1,000 genes and many are house-keeping genes (Ross et al. 2005). X chromosome inactivation (XCI) is a biological process utilized by mammals to ensure equal expression of X-linked genes between males and females (Lyon 1961). The inactive X chromosomes in the donor cell requires reactivation/reprogramming during NT and provides a very good marker for reprogramming studies. Interestingly, aberrant patterns of X chromosome inactivation were discovered in bovine clones (Xue et al. 2002), while in cloned mice both relatively normal (Eggan et al. 2000) and abnormal XCI patterns (Nolen et al. 2005; Senda et al. 2004) have been reported. In order to investigate the normality of XCI in cloned pigs, we selected five X-linked genes and one ubiquitous autosomal gene to compare their expression levels in major organs of cloned piglets and age-matched controls by quantitative real time RT-PCR. These genes are: 1) the untranslated X inactivation-specific transcript (*XIST*), which resides in the x-inactivation center (XIC) and serves as the master regulatory switch for X chromosome inactivation (Penny et al. 1996); 2) the noncoding antisense mRNA of *XIST*, *TSIX* (the reserve spelling of *XIST*), which regulates both imprinted and random XCI through modification of chromatin structure in the mouse (Lee 2000; Sado et al. 2005); 3) hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT1*), a housekeeping gene on X-chromosome and its deficiency causes Lesch-Nyhan Syndrome in humans resulting symptoms such as gouty arthritis and kidney/bladder stones; 4) glucose-6-phosphate dehydrogenase (*G6PD*), an enzyme involved in the hexose monophosphate pathway and its deficiency in the embryonic tissues impairs the development of the placenta, causing the death of the embryo (Longo et al. 2002); 5) V-raf murine sarcoma 3611 viral oncogene homolog 1 (*ARAF1*), a member of the raf proto-oncogene superfamily, which encodes cytoplasmic protein serine/threonine kinases and plays important roles in cell growth and development (Lee et al. 1994); and 6) Alpha-1 type IV collagen (*COL4A1*), located on porcine Chromosome 11, when mutated causes vascular defects in mice followed by Porcencephaly in some mutants (Gould et al. 2005). We found severe dysregulated expression of these genes in newborn deceased cloned piglets and moderate dysregulation in the surviving clones when compared to their respective age-matched controls.

Materials and Methods

Piglets and sample collection

All pigs in this study were females and fell in two age groups. Group 1 included six cloned pigs that died at or shortly after birth, and three controls from conventional reproduction that were healthy at birth and sacrificed for tissue collection. Four of the six NT-derived piglets were transgenic with one allele of the porcine $\alpha 1,3$ -galactosyltransferase ($\alpha 1,3$ GT) gene experimentally disrupted in the donor cells. The experimental protocols for knocking out this gene and generating these piglets have been described previously (Dai et al. 2002). The gestation period of these pigs ranged from 115 to 121 days. Minor phenotypic abnormalities, such as curled toes, were observed in three of the six cloned newborn pigs. These phenotypes have been observed occasionally in cloned piglets, and are not necessarily lethal. Piglets with these kinds of minor abnormalities have survived post-weaning and appeared to develop normally. For sample collection, deceased piglets were weighed, and tissues from major organs (heart, lung, liver, kidney, brain, spleen) were collected. Group 2 included seven cloned pigs derived from the same donor cell line and five age-matched controls. All pigs in Group 2 were euthanized at one month of age for the collection of tissues from the same six major organs. Tissues were snap frozen in liquid nitrogen and stored at -80°C until analysis. Animal handling and experimentation were in accordance with the National Research Council's publication "Guide for the Care and Use of Laboratory Animals" and approved by Institutional Animal Care and Use Committees at University of Missouri-Columbia and an equivalent organization at PPL Therapeutics, Inc. (currently Revivicor, Inc., Blacksburg, VA).

RNA preparation and reverse transcription (RT)

Total RNA from dissected tissues was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). RNA was then treated with the DNA-free Kit (Ambion, Austin, TX) to remove any possible DNA contamination. The complete removal of DNA contamination was confirmed by the absence of amplification of PCR products after 40 cycles using treated RNA as a template in the absence of RT. RNA was then quantified by absorbance at 260/280 nm using a spectrophotometer. Before RT, RNA quality was examined by ratios of A_{260}/A_{280} (all above 1.85 and mostly above 1.90) and by gel electrophoresis for the presence of two clear ribosomal RNA bands. Only RNA samples that did not show signs of degradation were used in this study. For RT, 1 μg of total RNA was added to a 20 μl -reaction mixture containing random hexamers and reverse transcriptase (New England Biolabs, Beverly, MA). The reaction mixture was incubated at 25°C for 10 min followed by RT at 42°C for 1 h. The reverse transcriptase was then inactivated at 95°C for 5 min.

Validation of the comparative C_T method for quantitative real time RT-PCR

Quantitative real time RT-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Reactions were performed in triplicates in 96-well optical reaction plates. Each reaction contained cDNA reverse transcribed from 10 ng total RNA, 1X SYBR Green PCR master mix and 0.3 μM of each specific primers, which were designed using Primer Express software (ABI) and summarized in Table 1. The T_m for each primer pair was between 59 and 60°C and the thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15s at 95°C for denaturation and 1 min at 60°C for annealing and extension. The fluorescence intensity for each well of the PCR plate was automatically determined after every elongation step. The specificity of the PCR reaction was confirmed by a single peak in the dissociation curve (Fig. 1a) and also by a single band in agarose gel electrophoreses (data not shown). Data were acquired and analyzed by ABI prism SDS software (ABI). The 18S ribosomal RNA was used as an endogenous control throughout this study. The calibrator, which was included in every real time plate, was a mixture of RNA from all six organs.

In order to use the comparative C_T method for quantification, also known as the $\Delta\Delta C_T$ method, each pair of primers was validated for equal amplification efficiency to primers of the endogenous reference (18S *rRNA*) at a wide range of cDNA concentrations. Briefly, serial dilutions of cDNA from mixed RNA samples were made, real time RT-PCR reactions for both the 18S *rRNA* and each target gene was performed in triplicates at each cDNA dilution (Fig. 1b). C_T , the threshold cycle, was determined after setting the threshold in the linear amplification phase of the PCR reaction and ΔC_T for a particular gene was defined as $C_T(\text{target gene}) - C_T(18S \text{ rRNA})$. As shown in Fig. 1c, a linear regression with a slope (absolute value) of less than 0.1 was obtained for each of the gene of interest, indicating that 18S *rRNA* and target genes had similar amplification efficiency which were close to 2.0 (Table 1), thus validating the quantitative nature of the RT-PCR reaction using the $\Delta\Delta C_T$ method. The relative expression level of a target gene in a particular sample was calculated as: $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$. The amplification efficiency was determined by the following formula: $e = 10^{-1/\text{slope}}$, where the slope is from the linear regression of the each gene's C_T values over the log values of serial dilutions of cDNA from 20, 5, 2.5, 1.25, 0.625, 0.3125 ng mixed RNA.

Distinguishing transcripts of *XIST* and *TSIX*

Because *XIST* and *TSIX* are antisense mRNA for each other, a regular RT reaction, in which random hexamers are used for reverse transcription, would convert both transcripts to the same double-stranded cDNA molecule. Therefore, strand-specific primers were used during RT and this was followed by real time PCR to determine mathematically the ratio of *XIST* and *TSIX*. The gene-specific RT primers for *XIST* and *TSIX* were 5'-AGGTGTTGCTGGCTGATGCT-3'; and 5'-GAAGAGATGCTCCAGCCAAT-3', respectively. These primers were subjected to reverse-phase cartridge purification and RT was conducted using Endofree RT kit (Ambion, Austin, TX) per manufacture's instruction to reduce the endogenous priming by fragmented oligonucleotides.

Statistical analysis

All statistical analyses were performed using SAS/STAT software (Version 8.2, Cary, NC). All data were first examined for distribution normality and variance equality, the latter of which was tested by the folded form of the F statistic (F') (Steel and Torrie 1980). Data with normal distribution and equal variance were subjected to pooled t-tests. Normally distributed data with unequal variance were subjected to the Cochran t-test. Data that were not normally distributed but have equal variance were tested using the Wilcoxon Rank Sum test. Data that were neither normally distributed nor equal in variance were log-transformed first and then tested as described above depending on their distribution and variance status. A p value of less than 0.05 was considered statistically significant.

Results

The mRNA levels of a total of five genes on the porcine X chromosome and one on the autosome were examined in our study and the results are summarized in Fig. 2 to Fig. 7. Comparisons of expression for each gene were made between controls and clones in a particular organ. For the neonate group, four comparisons were found to be significantly different between controls and clones among the 30 comparisons made. These include *HPRT1* expression in lung, brain and spleen, and *COL4A1* gene expression in kidney (Figs. 2a and 3a). While the other genes studied appear to have normal expression levels in all six organs. Interestingly, newborn deceased clones had lower levels of expression in all significant differences detected. When we compared the expression of each gene between controls and clones in the surviving group, five comparisons were found to be significantly different. All

of these were due to up-regulation in gene expression in the clones, including the expression of *XIST* in heart, lung and spleen, and *HPRT1* in lung and liver (Figs. 3b and 4b).

Relatively large variations in the expression levels of the genes studied were observed among different organs in newborns and this trend continued in one-month old pigs, regardless of their reproduction method. Interestingly, the most dramatic differences were found for the autosomal *COL4A1* gene, which were high in lung and kidney but very low in liver and brain in both aged groups. For the X-linked genes, the liver seemed to have the lowest *XIST* levels, while the brain had the highest *HPRT1* levels.

In addition, we tested homogeneity of variance and found six comparisons to have unequal variance in the clones when compared to the controls. These include *COL4A1* expression in heart (Fig. 2a), *HPRT1* expression in heart and kidney (Fig. 3a), *XIST* expression in brain (Fig. 4b), and *G6PD* expression in heart and kidney (Fig. 6a). Interestingly, larger variances were found in the clones for five out of six of these heterogeneous variances. When clones and controls were compared in groups, these large variances could mask dysregulation of individual clones and result in a lack of significant differences. Therefore, we used 2 standard deviations (2SD) of the expression level of controls as a cut-off to compare the cloned pigs individually, which gives us 95% confidence. Indeed, four genes, *XIST*, *HPRT1*, *COL4A1*, and *G6PD*, had more than 30% of parameters that varied more than 2SD of the control's values in the newborn deceased clones, while the remaining two genes studied were less variable (Table 2). On average, we found 36% of the 180 parameters (5 genes in each of 6 organs from 6 pigs = 180) determined in cloned newborn pigs that was dysregulated by at least 2SD of the control's values (Table 2). While in the live clones, the dysregulation was only moderate, 19% on average (Table 2). Interestingly, *XIST* appears to be the most deregulated gene in both the diseased newborn clones as well as clones at one-month of age, when these animals were analyzed individually.

To distinguish the *XIST* and *TSIX* transcripts, primers designed in the non-overlapping regions of these transcripts are necessary, as performed in the mouse and human (Lee, Migeon). Due to the lack of complete sequence information, we were unable to design such primers. Using strand-specific RT reactions should allow us to convert only the interested mRNA to cDNA. However, we were still able to detect positive band by gel electrophoresis (data not shown), suggesting endogenous priming was not completely eliminated despite the use of a system to reduce it. In order to circumvent this problem, we combined strand-specific RT and real time PCR in order to mathematically calculate the ratios of *XIST* and *TSIX* expression. We conducted these reactions in RNA extracts from all tissues and similar results were observed. Therefore, we pooled RNA (20 ng) from all six organs for strand-specific RT and real time PCR and only presented results from the pooled sample (Fig. 7). We found that the C_T values of RT-PCR with *TSIX* strand-specific primer and RT-PCR without primers (endogenous priming only) were the same. This indicated that there was no *TSIX* expression in these pig tissues at these ages.

Discussion

Abnormal expression of genes on the X-chromosome have been reported in cloned bovine embryos and full-term calves as well as in cloned mouse embryos (Wrenzycki et al. 2002; Xue et al. 2002). This is possibly due to the insufficient reprogramming of the donor cells and failure to reactivate/inactivate the X chromosome during embryonic development (Nolen et al. 2005). The present study is the first to investigate gene transcription levels in full-term piglets produced by somatic cell NT. Specifically, expression of five X-linked genes and one autosomal gene was studied. We found that while expression of these genes in surviving clones was largely within the normal range, abnormal expression was prevalent in deceased newborns.

Although porcine embryos from somatic cell nuclear transfer acquire a typical methylation pattern as control embryos and have normal telomere length (Jeon et al. 2005; Kang et al. 2001b), they display sporadic dysregulation of genes that are critical to embryo development (Lee et al. 2004; Miyazaki et al. 2005). This is consistent with the widely reported abnormal gene expression in mouse and bovine NT embryos (Bortvin et al. 2003; Daniels et al. 2000; Han et al. 2003; Mann et al. 2003; Wrenzycki et al. 2002; Wrenzycki et al. 2001). Different from the cloned pig embryos, however, bovine and murine cloned embryos also have abnormal DNA methylation patterns (Kang et al. 2001a), suggesting species differences in DNA methylation reprogramming. The wide-spread gene dysregulation in cloned embryos, regardless of species, may be a major contributor to the substantial loss of embryos through pregenancy. Because XCI occurs during early embryonic development, the abnormalities of X-linked gene expression observed in full-term piglets in the present study possibly persisted throughout fetal development, suggesting tolerance of these animals to dysregulated gene expression.

In the present study, we found that comparing clones to controls in groups is insufficient to discover the extent of expression anomalies. This is primarily due to the large degree of gene expression variation in the cloned animals, despite the fact that many of them were genetically identical to each other, for which a smaller variance would be expected. Using the 2SD of the normal expression as the cutoff, we found 65 out of 180 (36%) parameters in deceased newborns were abnormal vs. only 4 out of 30 (13%) parameters when clones and controls were compared in groups. In fact, the expression of all but one gene (*ARAF1*) in organs of individual deceased clones was found to exceed this threshold and was likely abnormal (Table 2). Similar expression abnormalities were found in DNA methylation in cloned pigs and deceased cloned bovine calves (Archer et al. 2003; Li et al., 2004; Yang et al., 2005). The same 2SD comparisons in the surviving clones revealed much less dysregulation (Table 2). This strongly suggests a correlation between the normality of gene expression and viability of the cloned piglets. Our finding was in accordance with a recent study in which the expression of several imprinted genes were found to be relatively normal in bovine clones that survived to adulthood compared to those that died at/after birth (Yang et al. 2005). Interestingly, all the above-mentioned studies, together with ours, reported more variation of gene expression in the cloned group, suggesting the universal random nature of nuclear reprogramming in different species.

We also noticed large variations in the expression levels of the same genes among different organs, regardless of how the animals were reproduced. This suggests that the expression of X-linked genes is not only regulated by XCI but also by their own individual promoters which may be subjected to tissue-specific stimulation/inhibition. The relatively consistent recapitulation of tissue specificity of gene expression in the cloned animals indicates that organ specific functions may be reprogrammed, despite the lack of complete reprogramming of XCI.

Finally, *TSIX* has been shown to repress *XIST* expression in cis and to regulate both imprinted and random X inactivation in mice (Lee 2000; Lee and Lu 1999). Unlike mice where *TSIX* expression is limited to undifferentiated cells, *TSIX* was reported to be present in human embryonic cell lines, fetal somatic cells and placental tissues, skin fibroblasts of 2-month old females (Chow et al. 2003; Migeon et al. 2001; Migeon et al. 2002), and even in adult bovine organs (Farazmand et al. 2004). These studies used *TSIX*-specific or strand-specific RT-PCR to distinguish the antisense transcripts of *TSIX* from *XIST*. Due to limited sequence information at the porcine *XIST/TSIX* locus, we were unable to design primers in non-overlapping regions of *TSIX* and *XIST*. The novel combination of strand-specific RT and quantitative real time PCR, however, allowed us to assertively conclude that *TSIX* was absent in our samples from newborn and one-month old pigs.

In summary, we document for the first time that cloned, deceased newborn pigs have prevalent abnormalities in the levels of X-linked genes, while clones that survived to one-month of age tend to have only moderate levels of expression abnormalities. Pigs do not express *TSIX* in major organs after birth.

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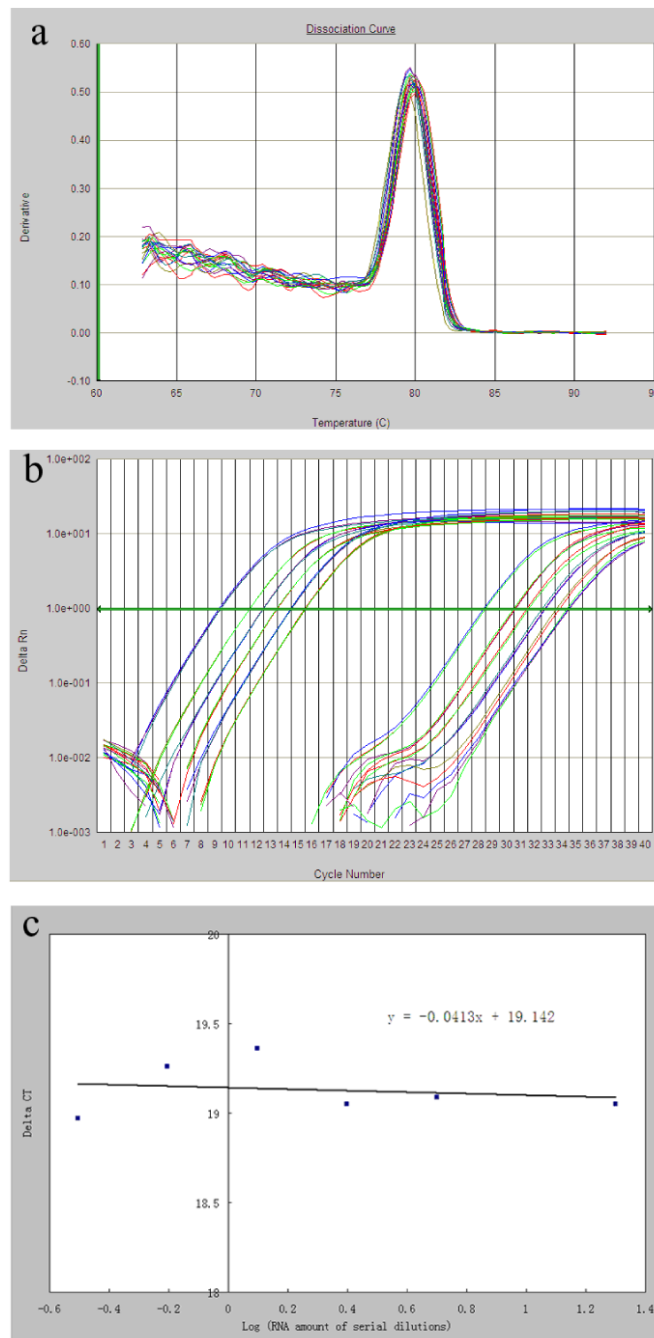


Fig. 1. Primer validation for quantitative real time RT-PCR using the comparative C_T method. a) A representative dissociation curve with a single peak, indicating the specificity of the amplification for the target gene (*G6PD*). b) A representative amplification plot of real time RT-PCR for the endogenous control, 18S *rRNA*, and the target gene (*G6PD*). The two sets of six curves for each gene (left set for 18S *rRNA*, right set for *G6PD*) represent serial cDNA dilutions of 20, 5, 2.5, 1.25, 0.625, 0.3125 ng (from left to right), respectively. The horizontal line in the middle was the threshold established for the calculation of the C_T value. c) A representative linear regression between ΔC_T , i.e. $C_{T(18S\ rRNA)} - C_{T(target\ gene)}$ and the log value of cDNA amount. The absolute value of the slope (0.0413) was smaller than 0.1, demonstrating

equal amplification efficiencies between the 18s *rRNA* (endogenous control) and the target gene (*G6PD*) within the cDNA dilutions tested.

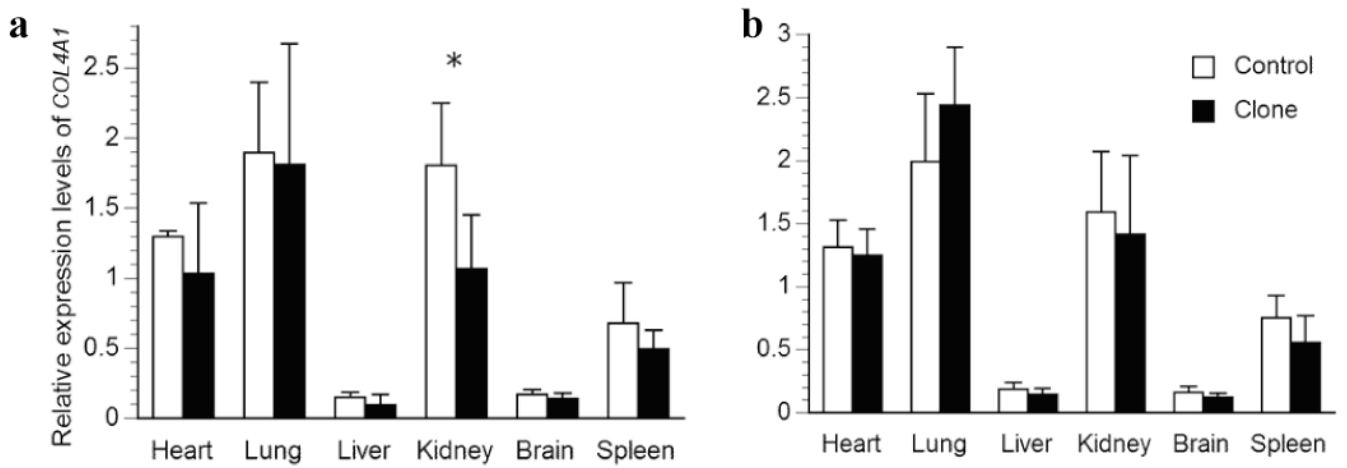


Fig. 2. Relative transcription levels for the autosomal *COL4A1* in major organs of a) deceased newborn clones or b) one-month-old surviving cloned piglets and their age-matched controls. The average transcription levels of each age group in the control piglets were normalized to 1. Bars with asterisks (*) are significantly different between clones and controls.

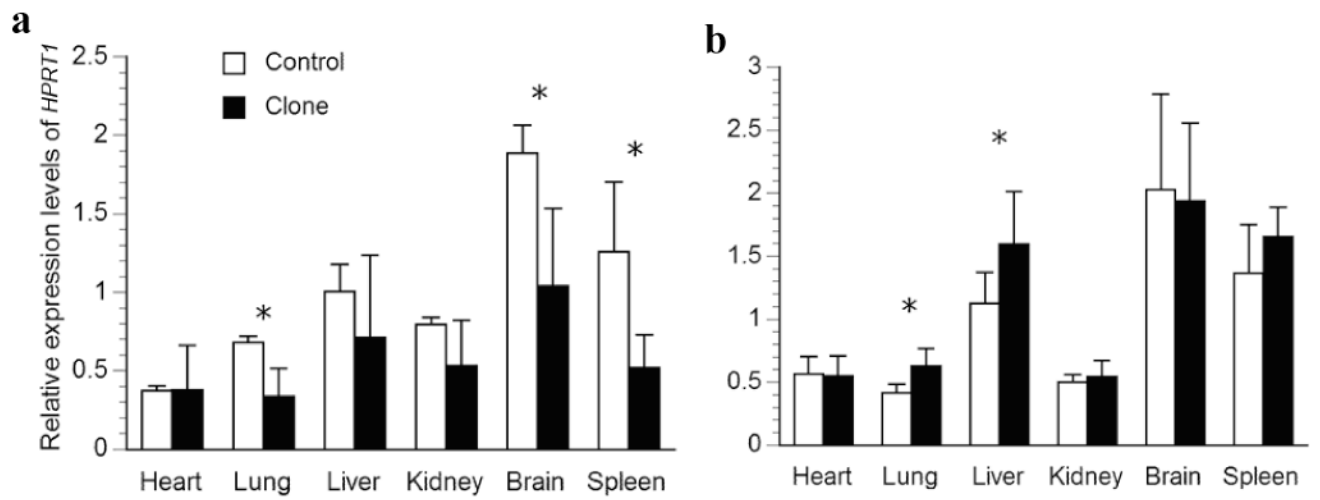


Fig. 3. Relative transcription levels for *HPRT1* in major organs of a) deceased newborn; b) one-month-old surviving cloned piglets and their age-matched controls. The average transcription levels of each age group in the control piglets were normalized to 1. Bars with asterisks (*) are significantly different between clones and controls.

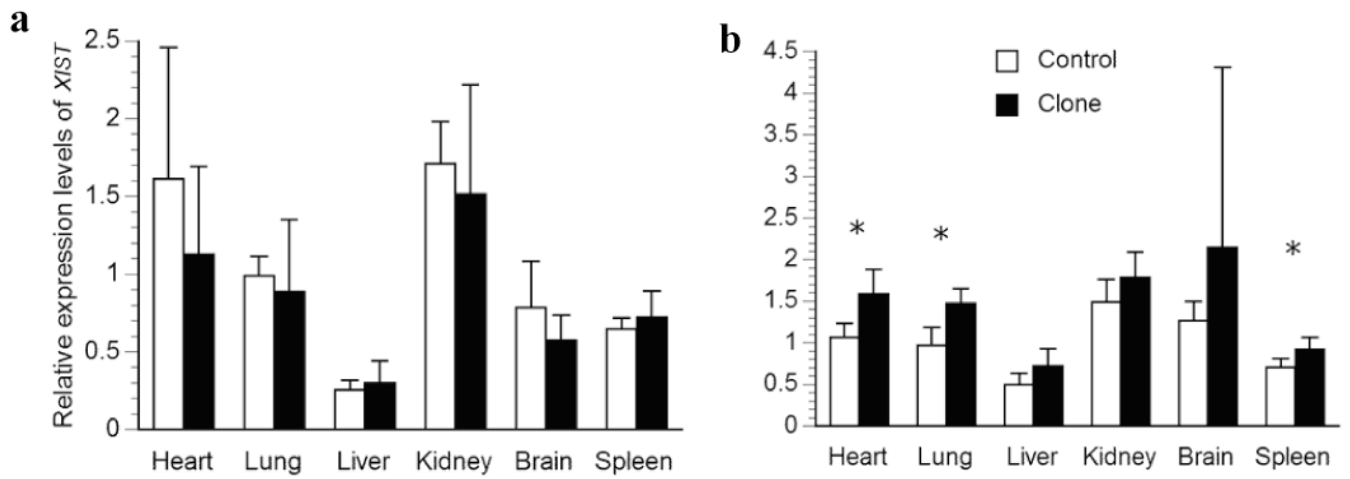


Fig. 4. Relative transcription levels for *XIST* in major organs of a) deceased newborn; b) one-month-old surviving cloned piglets and their age-matched controls. The average transcription levels of each age group in the control piglets were normalized to 1. Bars with asterisks (*) are significantly different between clones and controls.

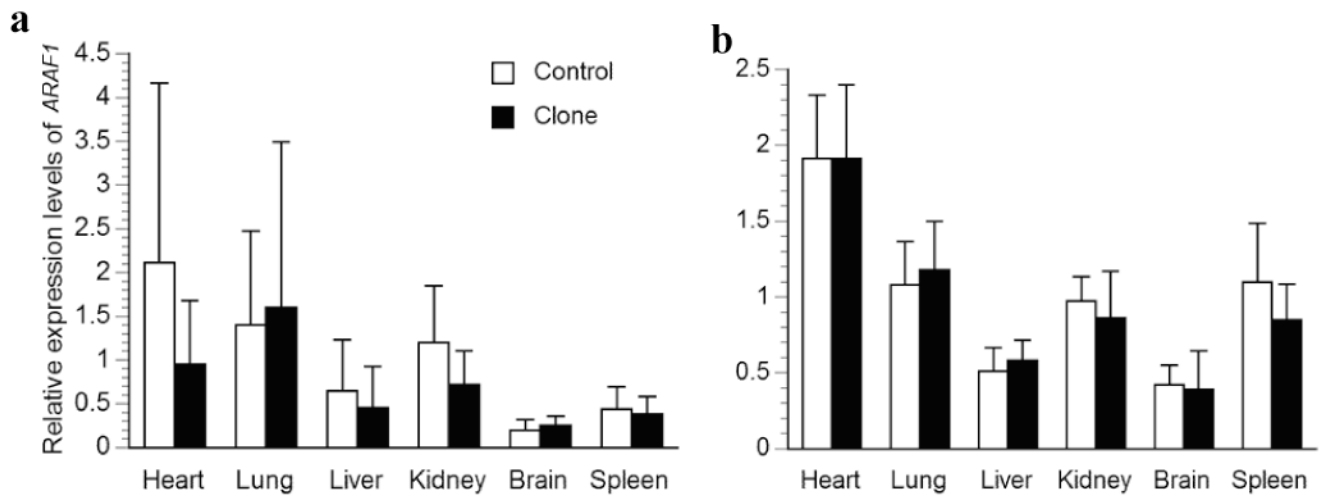


Fig. 5. Relative transcription levels for *ARAF1* in major organs of a) deceased newborn; b) one-month-old surviving cloned piglets and their age-matched controls. The average transcription levels of each age group in the control piglets were normalized to 1. Bars with asterisks (*) are significantly different between clones and controls.

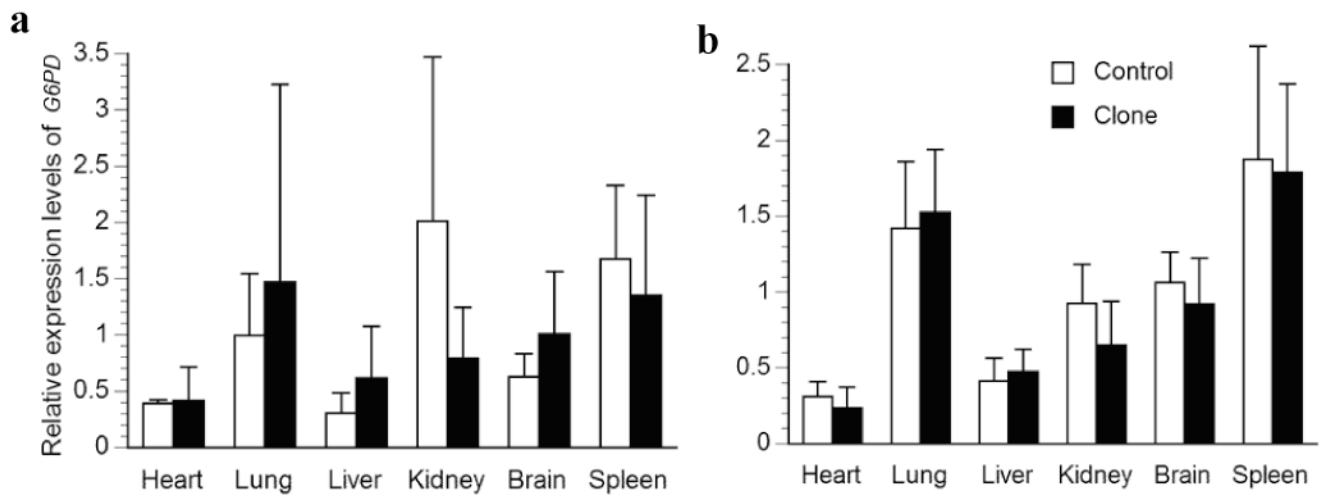


Fig. 6. Relative transcription levels for *G6PD* in major organs of a) deceased newborn; b) one-month-old surviving cloned piglets and their age-matched controls. The average transcription levels of each age group in the control piglets were normalized to 1. Bars with asterisks (*) are significantly different between clones and controls.

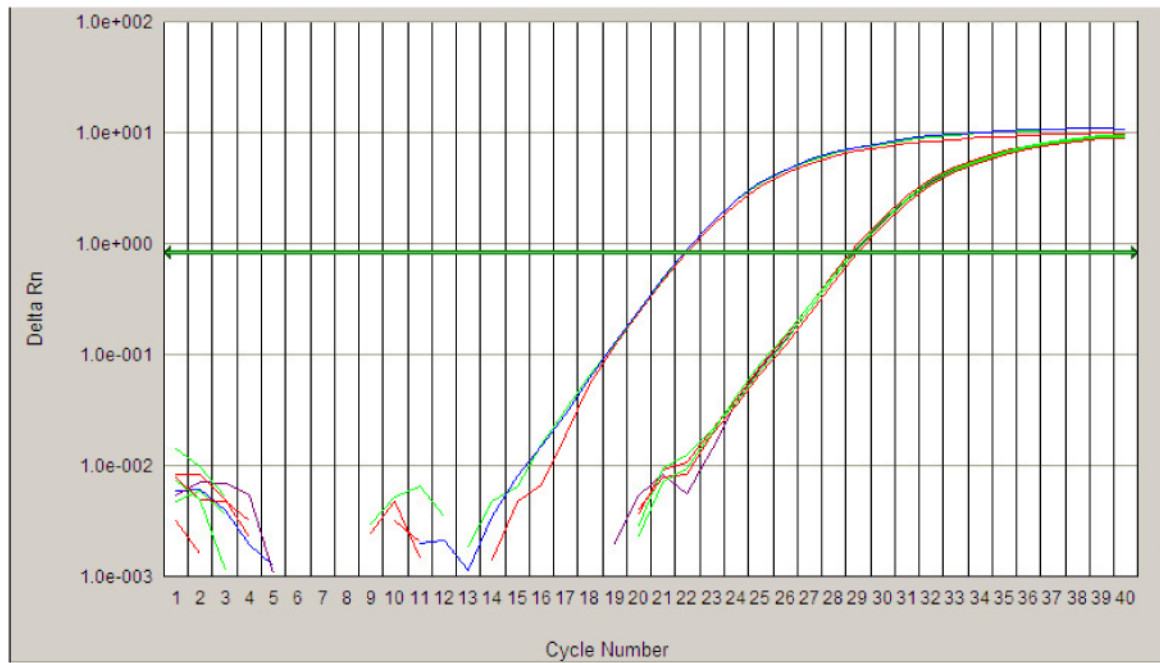


Fig. 7.

A quantitative real time amplification plot for the determination of the *TSIX* transcripts. The series of curves on the left represent triplicate amplifications of *XIST* using the *XIST*-specific primer. The series of curves on the right represent triplicate amplifications *TSIX* using either the *TSIX*-specific primer or no primers. The amplification curves for *TSIX* with and without primers overlapped, indicating a lack of *TSIX* transcripts in the samples. The same amount of total RNA was used in all amplifications in this figure.

Table 1

Primers for real time RT-PCR

Gene	Primer sequences (5' to 3')	Product size (bp)	Amplification efficiency
<i>XIST</i>	F: GAAGAGATGCTCCAGGCCAAT R: AGGTGTTGCTGGCTGATGCT	87	1.92
<i>HPRT11</i>	F: CGTCTTGCTCGAGATGTGATG R: TCCAGCAGGTCAGCAAAGAA	98	1.91
<i>G6PD</i>	F: CCTCCTGCAGATGCTGTGTCT R: CGCCTGCACCTCTGAGATG	112	1.91
<i>ARAF1</i>	F: CGGGATGGCATGAGTGTCTAC R: GACTGTCTTTCGCCCTTGA	108	1.98
<i>COL4A1</i>	F: CAGCAACGAACCCAGAAAT R: CAGCAAGAAGAGGCCAACAAG	120	1.89
18S <i>rRNA</i>	F: GCCCGAAGCGTTTACTTTGA R: CCGCGTCCTATTCCATTATT	93	1.96

F = Forward primer, R = Reverse primer.

Table 2
Percentage of gene expression dysregulated by at least 2SD of controls' expression

	XIST	HPRT11	G6PD	ARAF1	COL4A1
Dead Clones (n=6)	39%	72%	31%	3%	36%
Live Clones (n=7)	45%	17%	12%	17%	2%